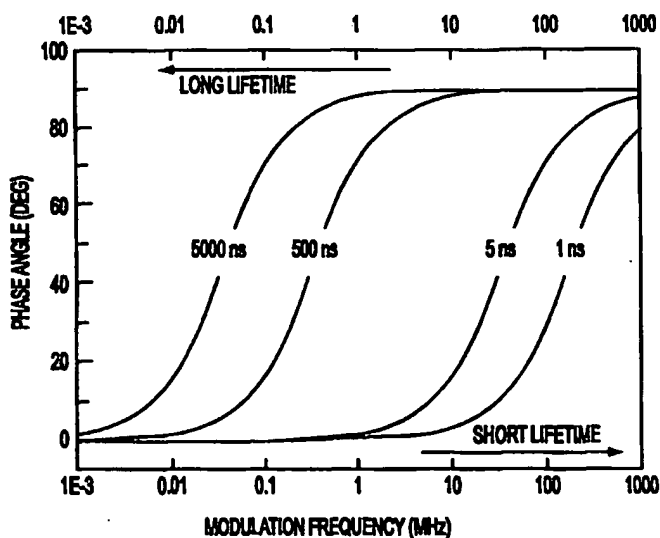




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>G01N 21/65</b>	<b>A1</b>	(11) International Publication Number: <b>WO 99/60385</b> (43) International Publication Date: 25 November 1999 (25.11.99)
(21) International Application Number: PCT/US99/11192 (22) International Filing Date: 20 May 1999 (20.05.99) (30) Priority Data: 60/086,174 20 May 1998 (20.05.98) US (71) Applicant (for all designated States except US): UNIVERSITY OF MARYLAND, BALTIMORE [US/US]; 520 West Lombard Street, Baltimore, MD 21201-1602 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LAKOWICZ, Joseph, R. [US/US]; 10037 Fox Den Road, Ellicott City, MD 21042 (US). SZMACINSKI, Henryk [US/US]; 8401 Glen Road, Baltimore, MD 21234 (US). (74) Agents: REPPER, George, R. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street N.W., Columbia Square, Washington, DC 20004 (US).	(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	

(54) Title: ANALYTE QUANTITATION USING A METAL-LIGAND COMPLEX PROBE



## (57) Abstract

Described is a new phase-modulation sensing scheme for a broad range of chemical optical sensing without requirements for lifetime-sensitive sensor dye. The measurement of concentration of an analyte relies on the measurement of phase angle or modulation of fluorescence of mixed fluorophores. The sensing probe consists of two fluorophores, a first fluorophore with the analyte-induced change in intensity and a second fluorophore which intensity does not appear to be sensitive to the analyte. The most important requirement for such sensing probe is that fluorescence lifetimes need to be significantly different. The new phase-modulation sensing scheme allows determination of fractional and total intensity of both dyes in a sample by measurement of the phase angle and modulation.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT -	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## ANALYTE QUANTITATION USING A METAL-LIGAND COMPLEX PROBE

**Introduction**

At present, there is considerable interest and research activity in the field of chemical sensing. Rapid and continuous monitoring of many analytes (pH,  $p\text{CO}_2$ ,  $\text{O}_2$ , metal ions, etc.) is required in many areas of science, including analytical chemistry, biochemistry, environmental sensing, clinical chemistry and industrial applications. Fluorescence-based sensing is one of the promising techniques because of fluorescence sensitivity, increasing number of sensitive and specific fluorescent probes for a variety of analytes (e.g. Molecular Probe Catalog) and their fabrication with fiber optics.

At present, most types of fluorescence sensing are based on the standard intensity-based methods, in which the intensity of the probe molecule changes in response to the analyte of interest. These intensity changes can be induced by an analyte due to changes in extinction coefficient, changes in quantum yield, absorption and emission spectral shifts, or simply due to the inner filter effects. While intensity measurements are simple and accurate in the laboratory, they are often inadequate in real-world situations. This is because the sample may be turbid, the optical surfaces may be imprecise and become dirty and optical alignment may vary from sample to sample. A significant disadvantage of intensity based sensing is the problem of referencing the intensity measurements. The fluorescence intensity measurement depends on the intensity of exciting light, the optical density at the excitation and emission wavelengths, the light losses in the optical path length, detector sensitivity and the concentration of the fluorophore. These difficulties with intensity-based sensing appear to be limiting the more widespread use of fluorescence for quantitative chemical sensing.

Recent advances in optoelectronic have now made possible a new type of fluorescence sensing. Instead of fluorescence intensities it is possible to measure fluorescence lifetime, particularly by the phase modulation method with a simple light sources. The advantages of lifetime-based sensing and the for a several mechanisms of analyte-induced changes in lifetime are reviewed elsewhere in detail (Szmajnski and Lakowicz, Topics in Fluorescence Spectroscopy, Vol 4, pp.295-334, Plenum Press, New York 1994). The preferred lifetime-based sensing technique is phase-modulation, where analyte-induced changes in lifetime of the probe are measured by phase angle and modulation at single modulation frequency. The phase and modulation are related to the analyte concentration. The number of various lifetime-sensitive probes were characterized for several analytes like pH,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ . Practically all of the known analyte lifetime-sensitive probes excluding the probes for  $\text{O}_2$  sensing display short lifetimes, most often in the range of 1-5 ns. Using the probes with short lifetimes require high modulation frequencies in the range of 50 - 300 MHz in order to obtain sufficient changes in phase and modulation for analyte sensing. However, in spite that inexpensive light sources like LED's can be modulated in that range of frequencies, the cost of phase-modulation device still seems to expensive because of requirement for high modulation frequencies.

There is observed a significant effort in several laboratories to develop the functional long lifetime probes in the range above 100 ns. There is a large number of fluorophores that display long lifetime fluorescence like metal-ligand complexes based on ruthenium, rhenium, osmium, platinum or rhodium. The lifetimes as long as 100  $\mu\text{s}$  can be obtained. However, there are not known MLC based probes that their lifetimes are sufficiently sensitive to analytes for practical use. Only  $\text{O}_2$  probes are used widely where the mechanism of quenching is exploited. Having a long lifetime probes the sensing can be at low modulation frequency in the range of 10-1000 kHz. The phase and/or modulation instrument can be designed based on inexpensive components. In addition in this range the LED's can be very easy modulated with high modulation depth.

The purpose of this invention is to use the advantages to measure phase and modulation in the low modulation frequencies and using available (or designed) fluorophores that intensity is sensitive to the analyte of interest. The one way to obtain this is mixing the analyte-intensity sensitive fluorophore with the second fluorophore that does not be analyte sensitive. It is known that the phase angle and modulation of the sample depend on values of lifetime and fractional intensities of components. The changes in phase angle and modulation can be as result of changes in fractional intensities without changes in the lifetime of both component. By mixing an analyte sensitive fluorophore (short lifetime or long lifetime) with the second fluorophore that does not be analyte sensitive (long lifetime or short lifetime) with controlled relative concentration, excitation wavelength and emission band an analyte sensitive probe can be created. The expected analyte induced changes in phase angle and modulation can be as large as 90 degree and 1.0, respectively. To observe large changes in phase angle and modulation the modulation frequency can be at lower range determined by the long lifetime component.

The controlled mixing of two fluorophores allows to turn any intensity-based fluorophore regardless of its lifetime as a lifetime-based probe using a phase and modulation technique. The analyte-induced changes in fractional intensities of two components allow to determine the analyte concentration from the phase and/or modulation at single modulation frequency. In addition, the absolute amount of fluorescence intensity from both dyes can be determined from phase and modulation measurements. This leads to opportunities to correct the intensity signal from probes in presence of background and autofluorescence.

The properties, requirements, advantages and various applications of the presented invention are discussed along with the drawings.

**Theory**

The total intensity decay is often described by a sum of exponentials

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

Where  $\alpha_i$  are the pre-exponential factors and  $\tau_i$  the decay times. The fractional intensity of each component is proportional to the pre-exponential factor and decay time,

$$f_i = \alpha_i \tau_i / \sum_j \alpha_j \tau_j \quad (2)$$

Consider the emission from a sample excited with a light modulated with circular frequency  $\omega$ . The frequency-dependent phase angle  $\phi_\omega$  and modulation  $m_\omega$  values can be calculated from the sine and cosine transforms of the impulse response. The necessary transform are

$$N_\omega = \frac{\int I(t) \sin \omega t dt}{\int I(t) dt} \quad (3)$$

$$D_\omega = \frac{\int I(t) \cos \omega t dt}{\int I(t) dt} \quad (4)$$

The analytical expressions for these transforms are give elsewhere (Lakowicz et al. Biophys. J. 46, 463, 1984 and Gratton et al., Biophys. J. 46, 478, 1982). The phase and modulation values are given by

$$\phi_\omega = \tan^{-1}(N_\omega/D_\omega) \quad (5)$$

$$m_\omega = (N_\omega^2 + D_\omega^2)^{1/2} \quad (6)$$

Let assume that total emission of the sample ( $I_{tot}$ ) consists of fluorescence with short intensity decay (S) and of long lifetime (L). The fractional intensity of short lifetime component is  $f_s$  and that of long lifetime component  $f_L$ , where  $f_L = 1 - f_s$ . At each frequency the total emission can be described by (Lakowicz and Balter, Biophys. Chem., 16, 99-115 and 117-132, 1982) by sine and cosine transform as

$$\begin{aligned} N_\omega &= f_s m_s \sin \phi_s + f_L m_L \sin \phi_L \\ D_\omega &= f_s m_s \cos \phi_s + f_L m_L \cos \phi_L \end{aligned} \quad (7)$$

Equations for phase angle and modulations can be derived by inserting the Eqs. (7) into the Eqs. (5) and (6). the equations for phase angle and modulation for a mixed sample can be obtained.

In this invention we are interested in cases where the difference between the short lifetime and long lifetime are in order of 50-fold or larger.

If the difference between the values of lifetimes are large than for certain range of modulation frequencies the phase angle and modulation of the mixed sample will be independent on the value of the short lifetime component because of  $\varphi_s = 0$  and  $m_s = 1.0$ . The analytical expression for phase angle and modulation in such cases are given as

$$\varphi_M = \tan^{-1} \frac{f_L m_L \sin \varphi_L}{1 - f_L + f_L m_L} \quad (8)$$

$$M_M = \sqrt{f_L^2 m_L^2 \sin^2 \varphi_L + (1 - f_L + f_L m_L)^2} \quad (9)$$

where  $\varphi_L = \tan^{-1}(\omega \tau_L)$  and  $m_L = (1 + \omega^2 \tau_L^2)^{-1/2}$ .

The phase angle and modulation of mixed sample will depend only on value of long lifetime component and its fractional intensity  $f_L$ . Thus, the phase angle measurement at single modulation frequency allows to determine the fractional intensities of both components and further calculate the amount of absolute signals from both species in at known total signal ( $I_m$ ) and the value of long lifetime component ( $\tau_L$ ).

Further simplification of Eqs. (8) and (9) can be obtained if the phase angle and modulation of mixed sample is measured at the frequency range where the modulation  $m_L$  is low, so the terms with  $m_L^2$  can be neglected. Straightforward relation between fractional intensity and modulation than is obtained

$$M_M = 1 - f_L \quad (9)$$

In other words the value of fluorescence modulation is equal the value of fractional intensity of short fluorescence component,  $f_s$ . Thus, the measurement of modulation of mixed fluorophores allows in certain conditions like large difference between values of long and short lifetime components directly measure the intensity of either components. It is important that in such cases there is no need to know the values of lifetimes in the sample. In frequency response of modulation we should observe steeples part within a certain range of modulation frequencies.

### Simulated Data

It is important to demonstrate the ability of above theoretical predictions. The more detailed description for simulated data one can find in part of Drawing Descriptions in Figs. 1a - 4a and Figs. 1b - 4b..

Fig. 1a and Fig. 1b show the expected frequency responses of phase angle and modulation for long lifetime and short lifetime fluorophores. Two distinct ranges of modulation frequencies are needed to measure the short lifetime and to measure long lifetime fluorescence. In order to measure the lifetime shorter than 5 ns that display most of organic fluorophores, high modulation frequencies are required in order of 100 MHz. This is achieved by an expensive phase-modulation fluorometers which are commonly used in the research labs. Long lifetime fluorescence requires low modulation frequencies in the range of 100 kHz. The design of phase-modulation instrument for low modulation frequencies is less expensive and can provide higher accuracy of measurements that similar with high frequency. However, there are not available lifetime-sensitive fluorescence sensors that display long lifetimes besides the sensing of oxygen using a metal-ligand complexes.

Fig. 2a and Fig 2b show the expected frequency responses of phase angle and modulation of fluorescence that consist of fraction of long lifetime and fraction the short lifetime fluorescence. The values from 0 to 1 represent the fractional intensity of short lifetime fluorescence in the measured signal. There are observed great changes from 0 to about 90 deg in phase angle and from 1 to 0 in modulation values upon changes of fractional contribution of fluorescence from both fluorophores. In addition the steeples part of modulation value is equal the fractional intensity of short lifetime fluorescence. Thus fractional-dependent phase angle and/or modulation can be used to measure the intensity of desired fluorophore in the sample using in most cases only one modulation frequency. The changes in fractional intensity can be induced by the analyte; (1) by affecting the absorption spectra (extinction coefficient and/or spectra shift), (2) by affecting the emission spectra (quantum yields and/or spectra shifts). There are many possibilities to optimize such sensor probe by the choice of excitation wavelength, emission band and relative concentration of used fluorophores.

Fig. 3a and Fig. 3b show the expected frequency responses of phase angle and modulation where the value of short lifetime is changed from 0.5 to 10 ns in several steps. The fractional intensity of short component in each case is the same of 0.15 in Fig. 3a and 0.5 in Fig.3b. The important observation from these figures are that the phase angle and modulation below certain frequency are not sensitive to the value of short lifetime fluorescence in the sample. This is similar to the gating technique in pulse method where by applying a certain delay after pulse excitation only the signal from long lifetime fluorescence is detected. In phase-modulation technique it is impossible to measure only long lifetime component. The analytical methods have been developed for background correction in phase-modulation fluorometry based on the measurements of the background sample or based on known intensity decay of background and its contribution in the sample signal. In the case presented in Fig. 3a and 3b the desired intensity of long lifetime component can be obtained by measuring the phase and/or modulation at single modulation frequency regardless of intensity decay of the background or autofluorescence or scattered light until mean lifetime is short enough compare to long lifetime

fluorophore. This feature can be used also to determine the anisotropy of long lifetime component in turbid media with scattered light or with background fluorescence with known value of anisotropy. This may find immediate application in detecting binding of high molecular weight macromolecules labeled with a metal-ligand complexes or in immunoassays. It is also important for chemical sensing that changes in the lifetime due to analyte for short lifetime indicators have no effect of fractional intensity and thus on sensing of analyte concentration.

Fig. 4a and Fig 4b show the expected frequency responses of phase angle and modulation where the value of long lifetime is 100, 500, and 5000 ns and short lifetime fluorescence of 10 ns. The most important observation is from Fig 4b where the value of steeples part of modulation reflect the fractional intensity of short lifetime component regardless on the value of the long lifetime component. Also is important that the choice of low modulation frequency depends mostly on the value of long lifetime component but not on the value of short component.

## Experimental Data

There three experimental examples to demonstrate the described invention. The Example 1 demonstrate the phase and modulation sensitivity when the fractional intensity of sample are varied by various relative concentration of two dyes in the sample.

The Example 2 demonstrates the possibility to determine the intensity of fluorophore of interest in presence of various amount of background or autofluorescence from the solvent.

The Example 3 demonstrate how the sensing probe can be created when pH induced changes in fractional intensities of a probe contained pH intensity sensitive indicator and long lifetime fluorophore can be measured by phase angle and modulation.

### Example 1

Two fluorophores have been chosen, one with a long lifetime fluorescence from metal-ligand complexes like  $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$  with a lifetime in glycerol of 1060 ns and the second with short lifetime like many organic fluorophores Texas Red Hydrazide with a lifetime of 3.4 ns in glycerol. The two dyes were mixed at various relative concentrations to induce the various fractional intensities in the sample

Fig. 5a show the absorption spectra of long lifetime fluorophore  $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$  and short lifetime fluorophore Texas Red Hydrazide (TRH) (solid lines and their mixture at concentrations specified in Figure. It is shown that any excitation wavelength shorter than about 640 nm will excite both fluorophores. The resulting fractional intensities from both fluorophores will be strongly dependent on the choice of excitation wavelength. One can imagine that value of extinction coefficient or shift in absorption spectrum will result in changes of fractional intensities that can be monitored with phase and/or modulation measurements. One excitation wavelength has been chosen as 488 nm (Argon-ion laser). The total concentration of dyes were low to avoid the inner filter effects. The changes in absorption was induced by using various concentration combination of both fluorophores.

Fig. 5b shows the emission spectra of  $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$  and TRH at one selected concentration combination. The emission spectra overlap well and for phase and modulation measurements we used the long pass filter above 550 nm.

Fig. 6 show the frequency responses of phase angle for long lifetime fluorophore  $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$  with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative concentrations from 0 to 12.8. The obtained values for fractional intensities are in good agreement with those expected from steady-state measurements of full emission spectra. These experimental data are related to those simulated and discussed in Fig. 2a.

Fig. 7 show the frequency responses of modulation for long lifetime fluorophore  $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$  with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative concentrations from 0 to 12.8. These experimental data confirm that presented and discussed in Fig. 2b.

### Example 2

The purpose of this example was to demonstrate the calculation of intensity of long lifetime fluorophore in presence of background fluorescence from the solvent. Long lifetime fluorophore was the same as in Example 1  $[\text{Ru}(\text{bpy})_3\text{dcbpy}]^2$  with a lifetime in glycerol of 1060 ns. The glycerol (from Fluka) displayed a background fluorescence that overlaps with the emission of ruthenium. In many applications the requirements are for very low dye concentration which poses the difficulties for increased background corrections. The increased contribution of background fluorescence from solvent was obtained by the dilutions of the ruthenium sample with glycerol.

Fig. 8 show the emission spectra of ruthenium with decreased concentrations and also background fluorescence from used glycerol. The fractional intensity of glycerol calculated by integrating the spectra are following: 0.108, 0.379, 0.757, and 0.886 at ruthenium concentration of 740, 150, 29 and 6 nM.

Fig. 9 show frequency responses of phase angle of the samples with increased contributions of background fluorescence. The obtained values are in good agreement with those from steady-state measurements. The small difference are because of different excitation sources (xenon lamp and monochromator in steady-state, and Ar-ion laser in phase-modulation measurements). It should be noted that phase angle is related only to fractional intensity at modulation frequencies lower than 1 MHz. The glycerol displayed a complex intensity decay with a mean lifetime shorter than 3.5 ns. These experimental data confirm that presented in Fig 3a where the short lifetime component do not contribute to changes in phase angle for certain low modulation frequencies.

Fig 10 show frequency responses of modulation of the samples with increased contributions of background fluorescence. The steeples part of modulation indicate good separation between the fluorescence of ruthenium and that of glycerol and can be easy used to determine the absolute intensity of the ruthenium. These results confirm that discussed in Fig. 3b.

### Example 3

The goal of this example is to demonstrate the great opportunity of designing the fluorescence probe for measuring a large variety of chemical species where the change in fluorescence intensity can be obtained. For example we have chosen a pH intensity sensitive indicator Naphtofluorescein and the second dye with a long lifetime  $[\text{Ru}(\text{pln})_3]^{2+}$ . The Naphtofluorescein as most of fluorescein dyes display pH sensitive absorption spectrum and decreased fluorescence quantum yield at lower values of pH. To demonstrate the practical use of such sensor we used inexpensive blue LED as a excitation source.

Fig. 11 shows the emission spectra of a mixture of ruthenium and Naphtofluorescein at various values of pH. The increased pH values affect the fractional intensities from both of dyes which is displayed as decreased fluorescence from the ruthenium and increased contribution from the Naphtofluorescein. The fractional intensities in the sample can be selected by the cutt off filter or by band pass filter. We have chosen use long pass filter above the 595 nm. The excitation source was a blue LED with a

maximum intensity at 475 nm.

Fig. 12 shows the frequency responses of phase angle of such pH sensor. There are observed remarkably large changes in phase angle at modulation induced by the pH of a sample. The pH phase-based sensing can be performed at low modulation frequencies in spite of very short lifetime of Naphtofluorescein of about 0.45 ns frequencies below 10 MHz.

Fig. 13 shows large changes in modulation induced by pH of the sample. There is a wide range of modulation frequencies where modulation value is related only to the pH value even not to modulation frequency. This is because the difference in lifetimes of ruthenium and Naphtofluorescein is very large about 1000-fold. It is again important to note that long lifetime value determines the useful low modulation frequency for sensing.

Fig. 14 shows pH-dependent phase angle for several modulation frequencies. It should be noted the magnitude of phase angle changes up to 69 deg (see values in the brackets) . This is remarkably pH sensor, which allows measurements the pH changes as small as of 0.0035 of pH unit assuming that phase angle can be measured with an accuracy of 0.1 deg (from curve at 2200 kHz in the range from pH 6 to 8) . Also choosing the modulation frequency allow to shift the apparent pKa, in presented case from 6.41 to 7.24.

Fig. 15 shows pH-dependent modulations for several modulation frequencies. The pH induced changes in modulation ( values in the brackets) are large and significantly depends on the choice of modulation frequency. The apparent pKa is slightly dependent on modulation frequency.

**Applications of the Invention**

1. Can be applied to correct the intensity signal in presence of scattered light.
2. Can be applied to correct the intensity in presence of background fluorescence and autofluorescence.
3. Can be applied to measure the analyte induced intensity changes of fluorescence probes by the phase and modulation measurements. The concentration of the analyte is related to the values of measure phase angle and modulation. The analyte intensity sensitive dyes need be mixed with the second dye which does not be sensitive to the analyte. The difference between values of lifetimes of used two components is preferable larger than 50-fold.
4. Can be applied for correction of anisotropy of fluorophore of interest in the samples with the background autofluorescence. Three modes are regarded as important (1) when long lifetime anisotropy probe is used and background correction is needed because of short lifetime background autofluorescence, (2) the long lifetime component is intentionally added in purpose to obtain information about changes in the background autofluorescence, (3) the long lifetime component is added to monitor very small changes in intensity of short lifetime probe. In all three modes the phase angle and modulation values are used to obtain the information about the intensity changes.

This invention is based on the discovery that, at the modulation frequencies at which, first, the short lifetime component results in a phase angle near zero and modulation close to one and, secondly, the long lifetime component results in a phase angle close to 90 degrees and modulation is close to zero, one can measure directly the fractional intensity of mixed fluorophors in order to determine signal fluorescence in the presence background fluorescence.

**CLAIMS**

- 1 1. A method of determining the concentration of an analyte in a liquid, comprising:
  - 2 a) adding a probe to the liquid to form a composition containing a mixture of
  - 3 fluorophores;
  - 4 b) exciting the composition with radiation so as to result in a mixture of fluorescent
  - 5 signals including a signal of interest indicative of the concentration of said
  - 6 analyte, and further including background signal information;
  - 7 c) identifying the signal of interest indicative of said analyte concentration; and
  - 8 d) determining the concentration of said analyte based on said signal of interest.
- 1 2. The method of claim 1 wherein said probe is a metal-ligand complex probe.

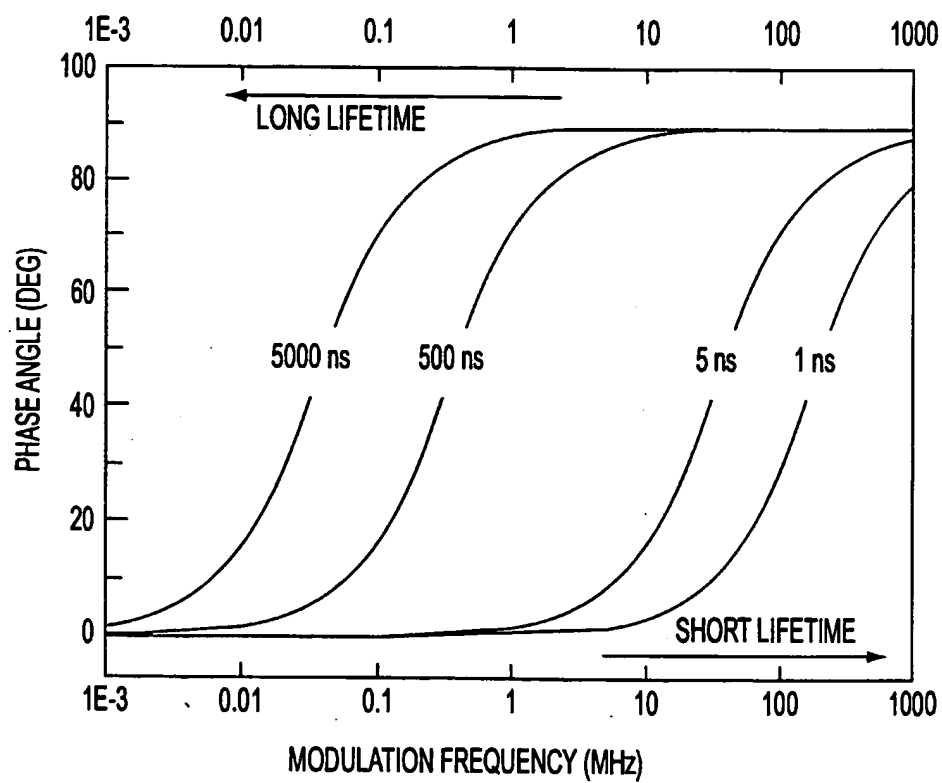


FIG. 1A

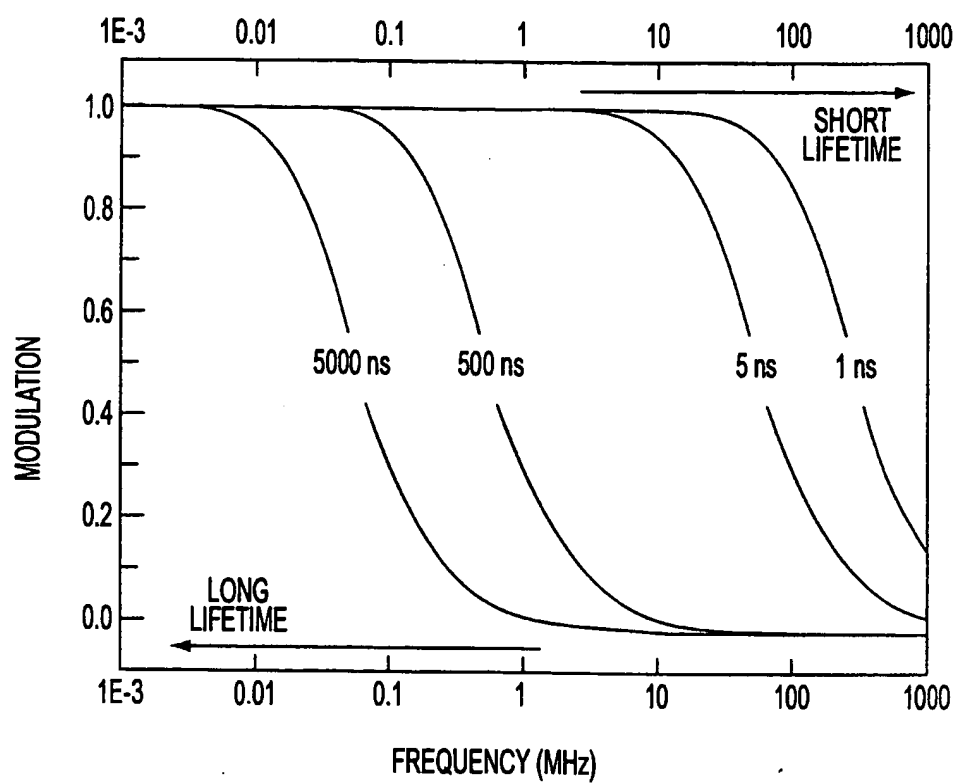


FIG. 1B

3/20

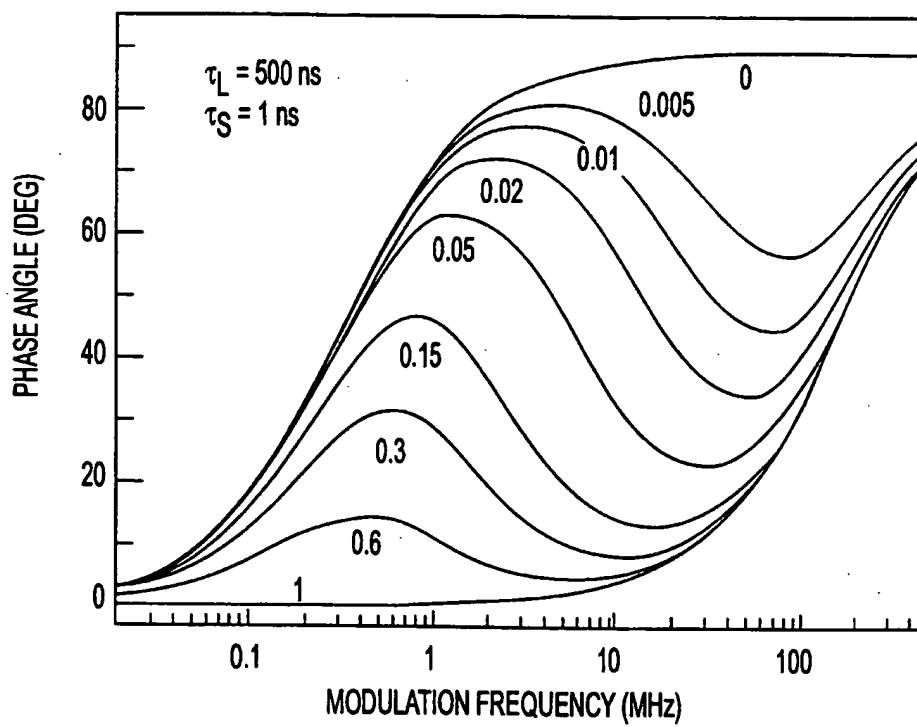


FIG. 2A

4/20

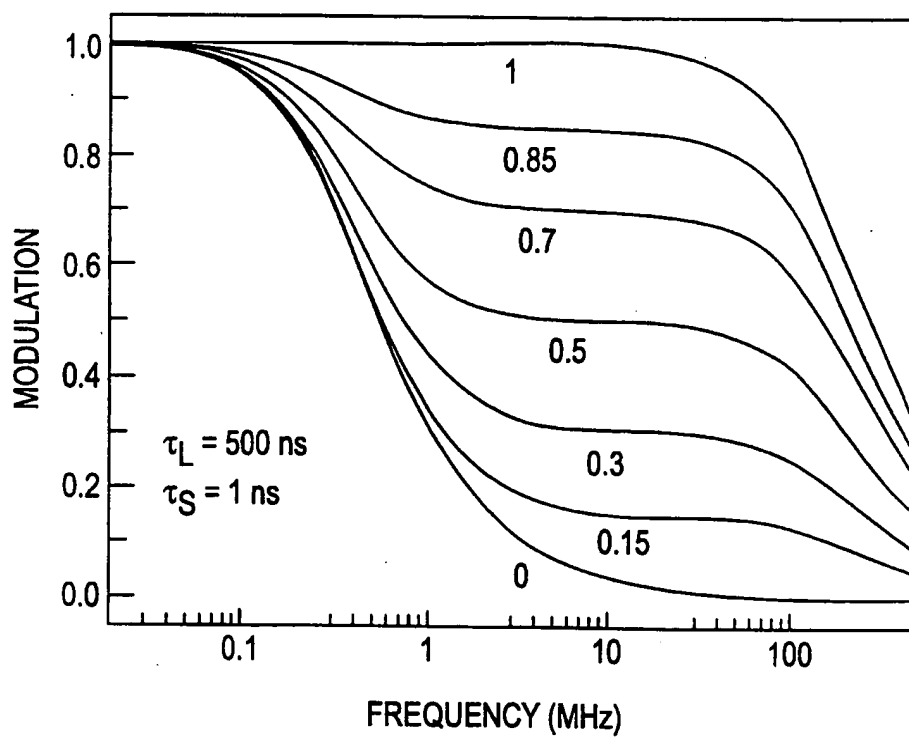


FIG. 2B

5/20

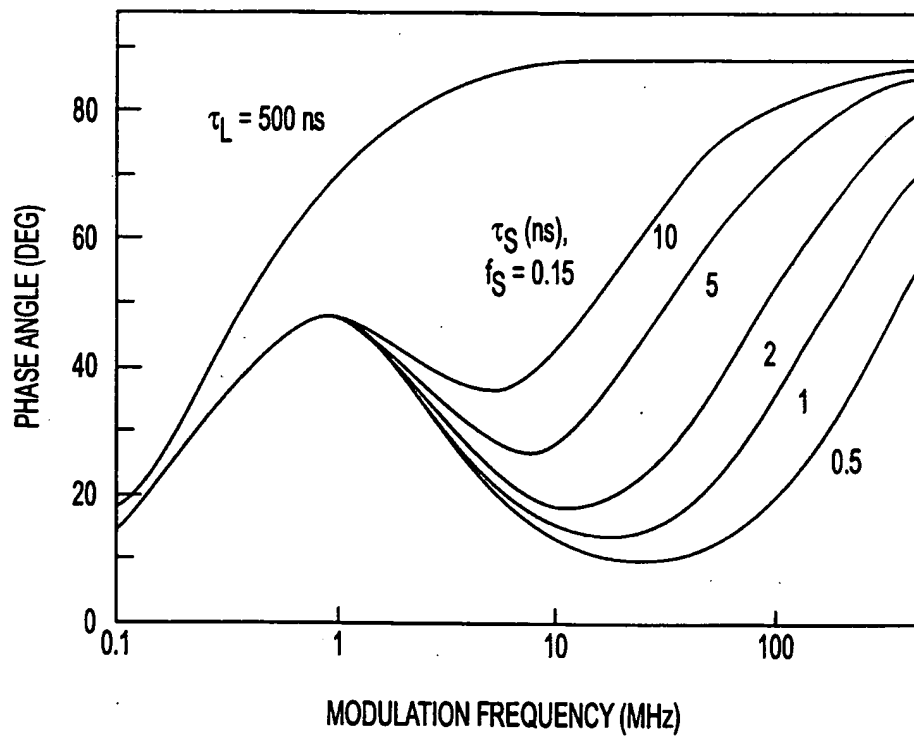


FIG. 3A

6/20

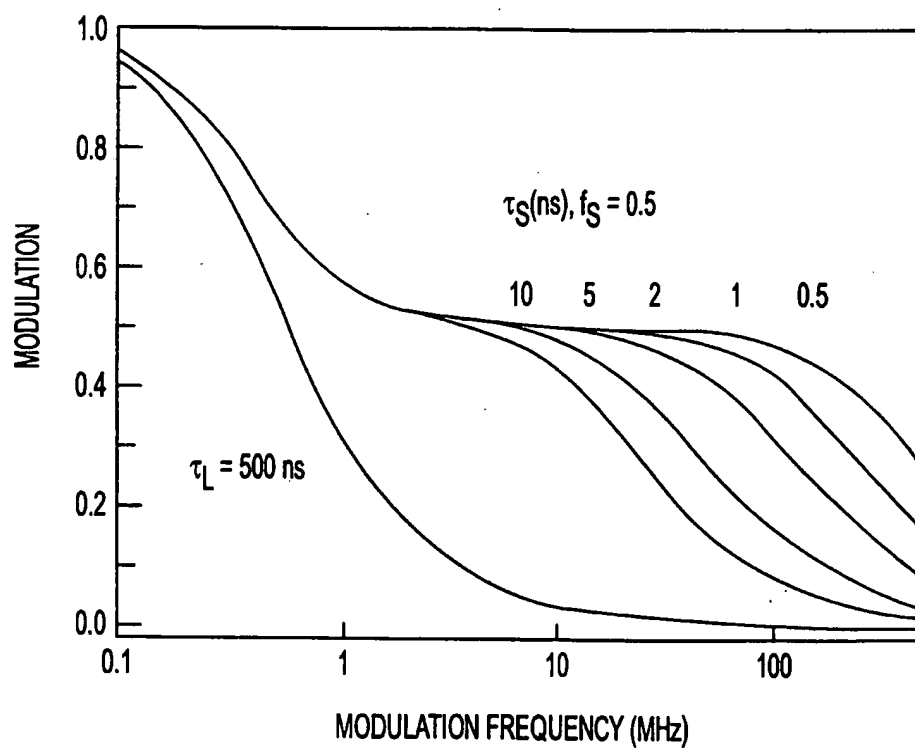


FIG. 3B

7/20

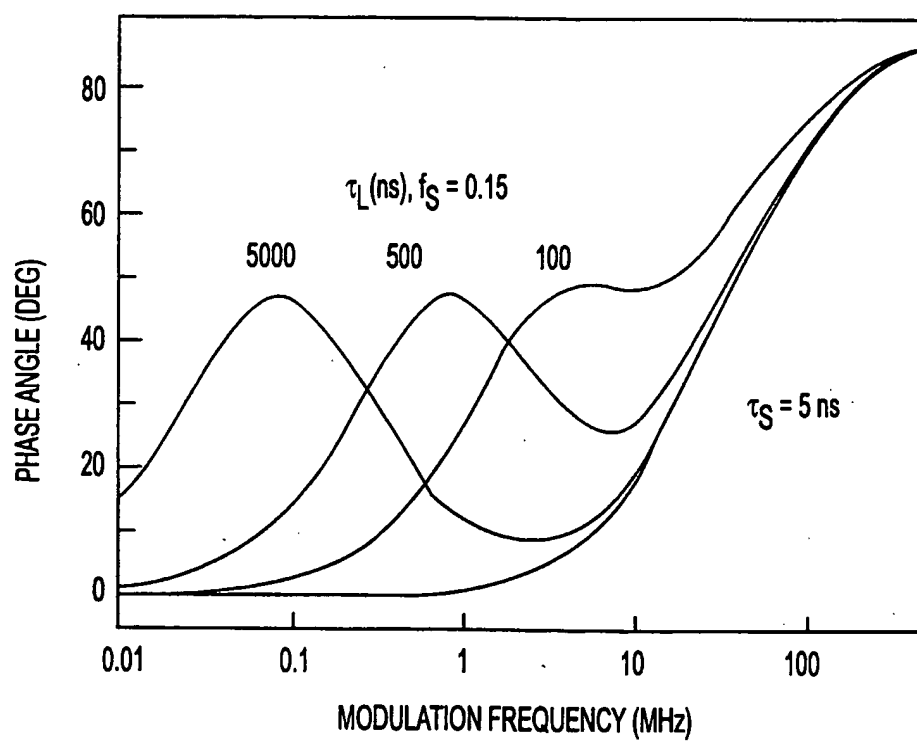


FIG. 4A

8/20

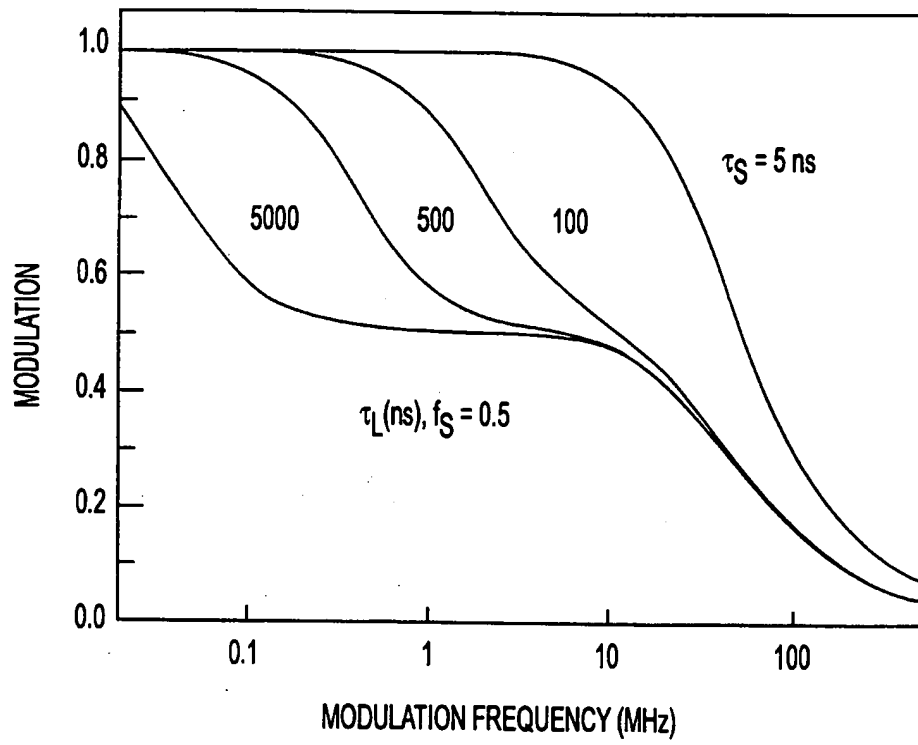
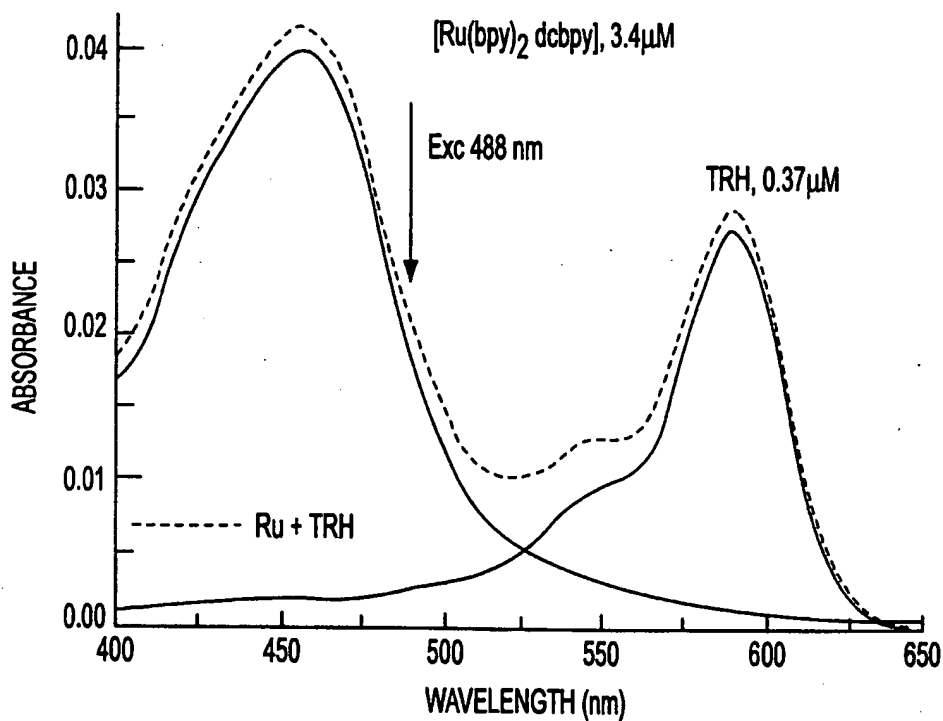


FIG. 4B

9/20



SERIE B

OD Ru = 0.21

TRH = 0.0024 (488NM)

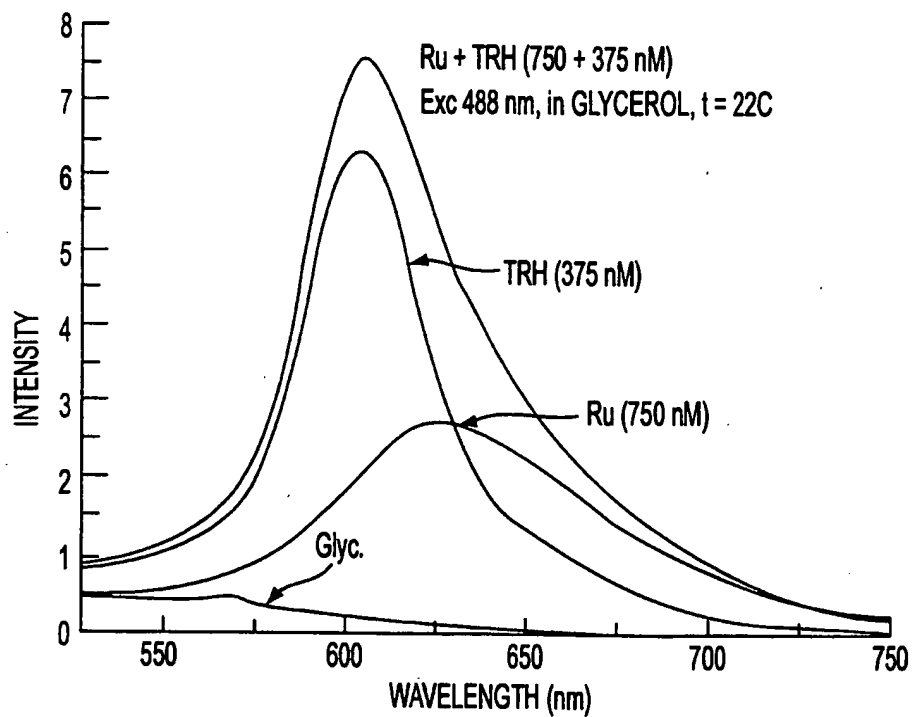
DYES WERE IN GLYCEROL (FLUKA).

CONCENTRATION CALCULATED BASE ON EXTINCTION COEFFICIENTS:

Ru -  $12,000 \text{ M}^{-1}\text{cm}^{-1}$ , [Evald]TRH -  $80,000 \text{ M}^{-1}\text{cm}^{-1}$  [MP]

FIG. 5A

10/20



FRACTIONAL INTENSITY IN THE MIXTURE (SERIE C):

GLYC.	- 0.065				
Ru	- 0.439	} .417	OD = 0.0042	/ YUT 1.41*	} Ø = 2.47*
TRH	- 0.613				

IF SEPARATELY IT IS ABOUT 11% MORE THAN IN MIXTURE.

FIG. 5B

11/20

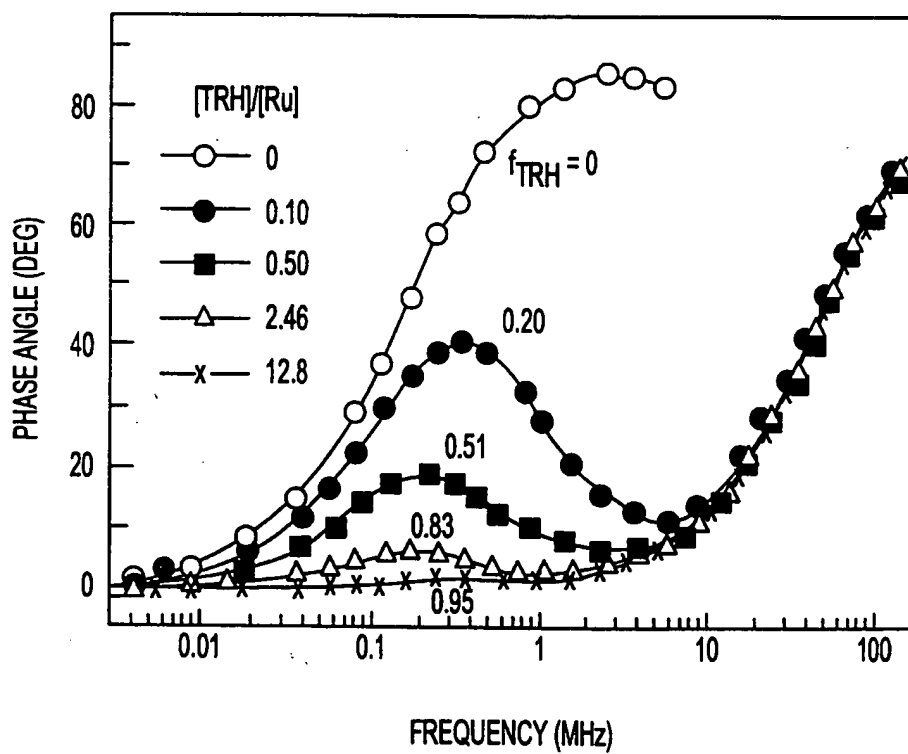


FIG. 6

12/20

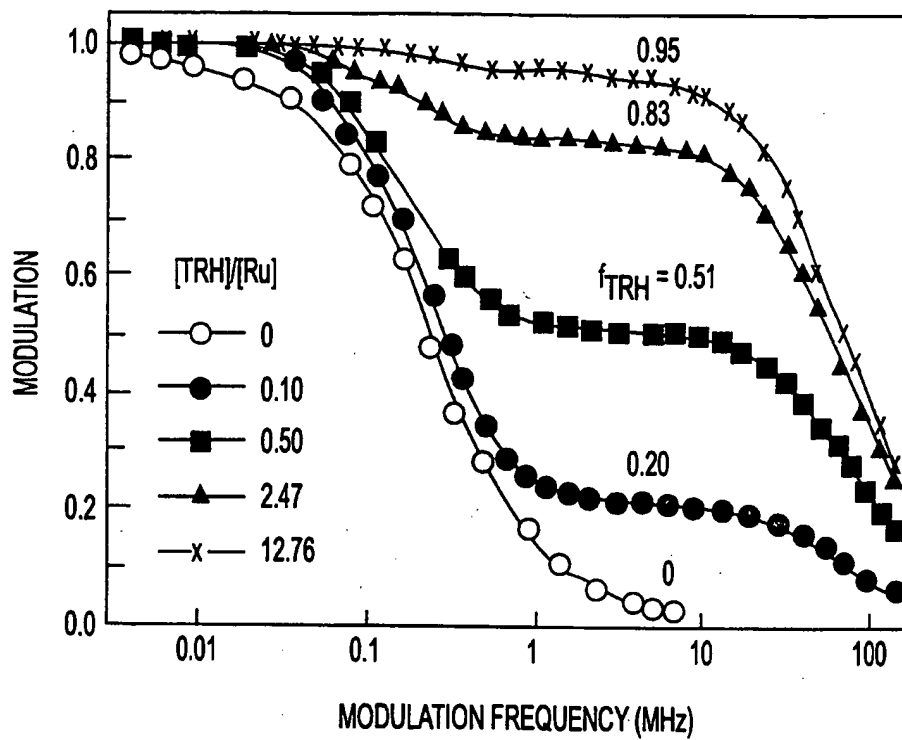


FIG. 7

13/20

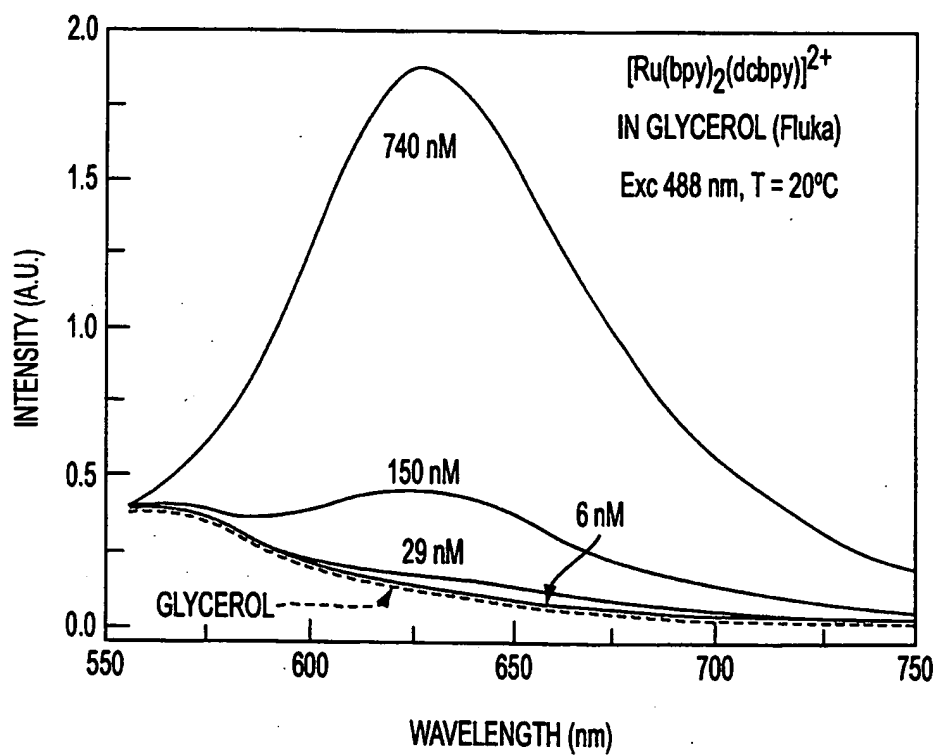


FIG. 8

14/20

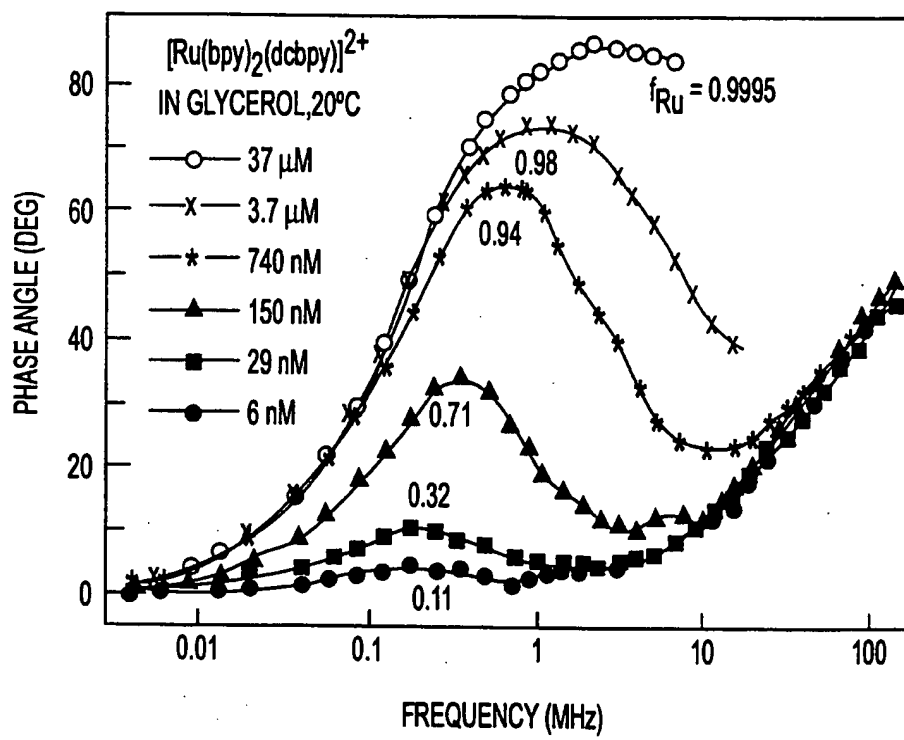


FIG.9

15/20

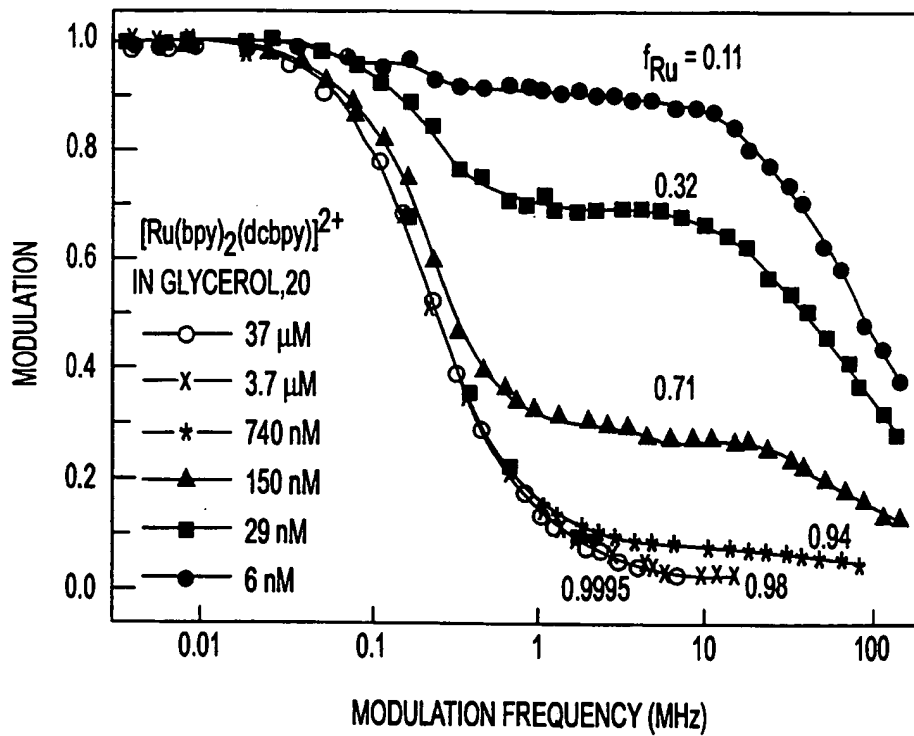


FIG. 10

16/20

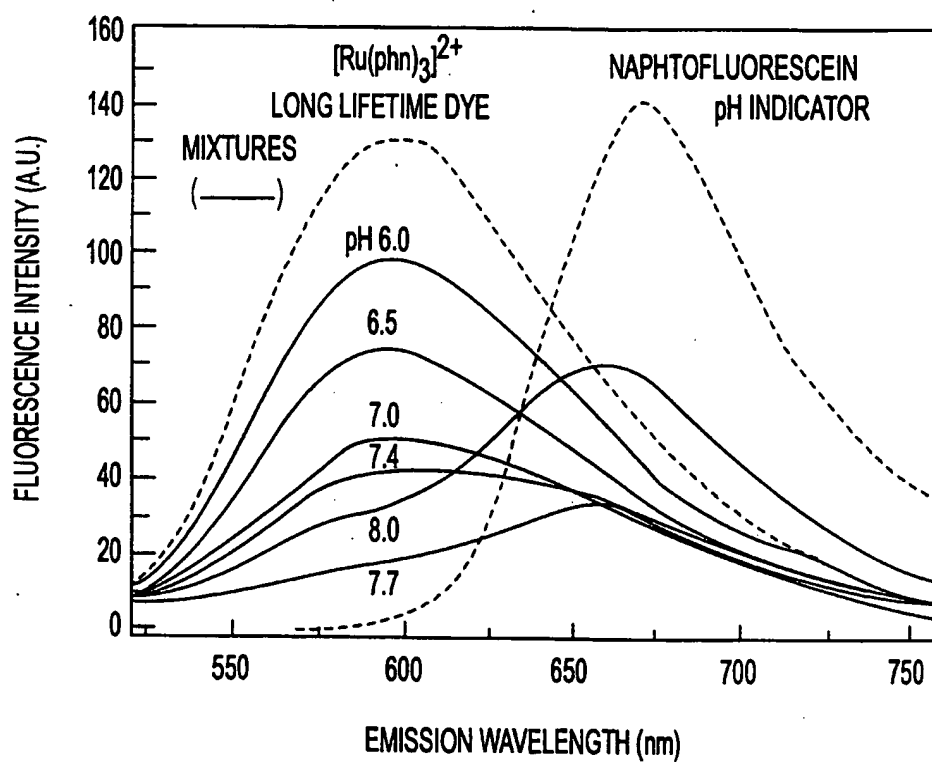


FIG. 11

17/20

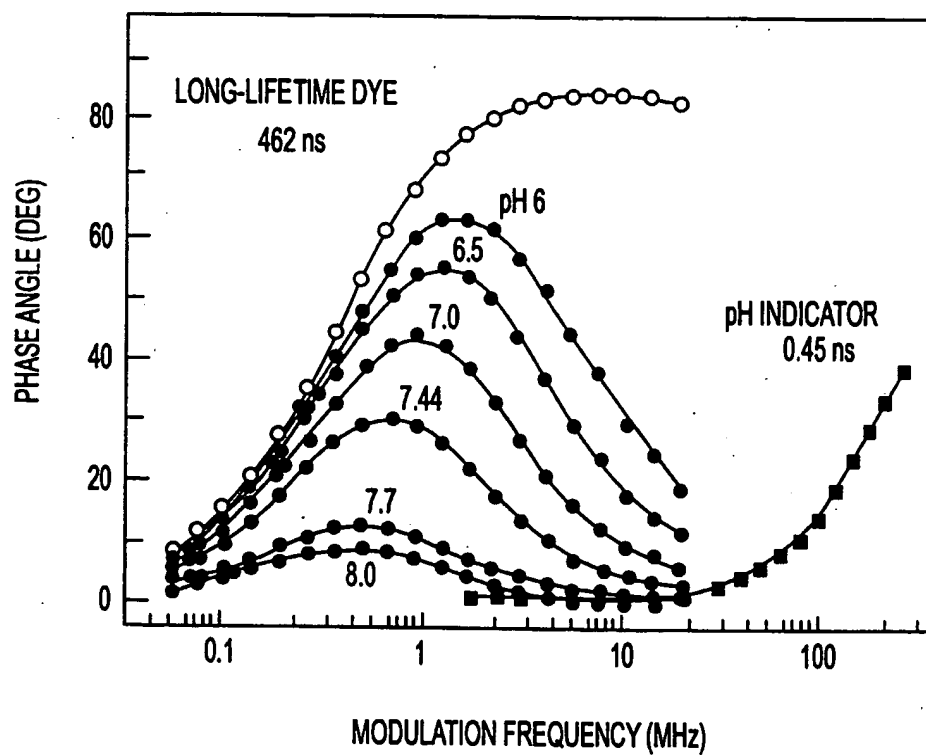


FIG. 12

18/20

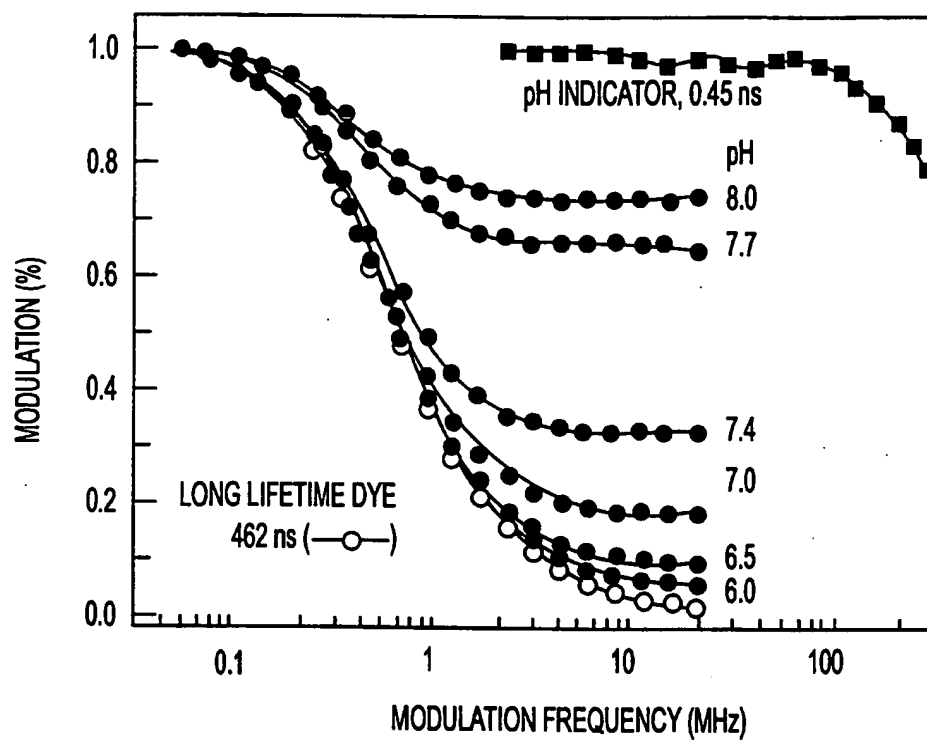


FIG. 13

19/20

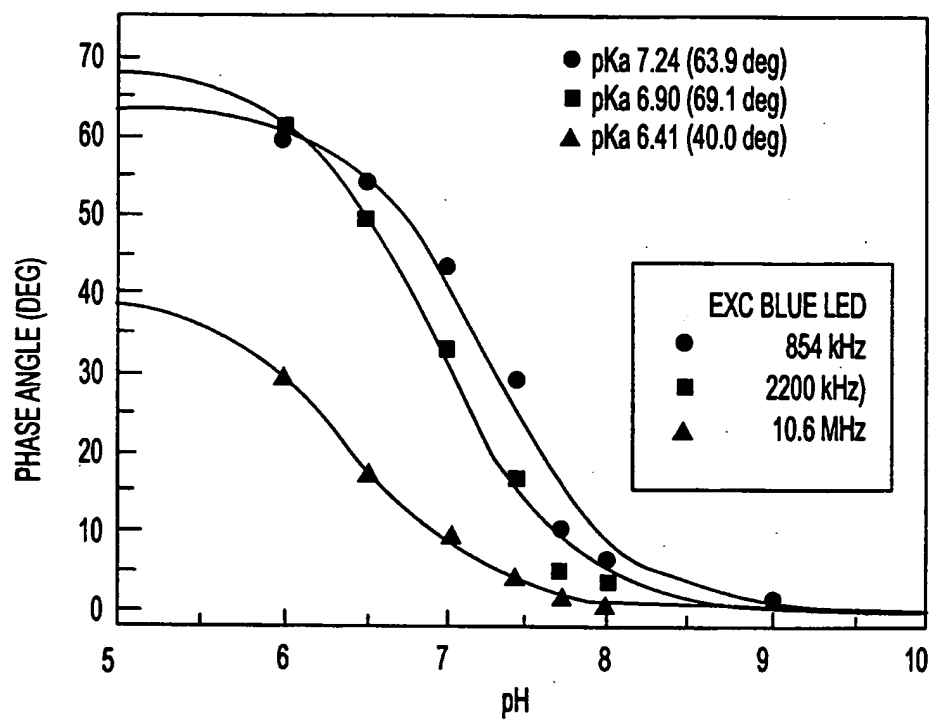


FIG. 14

20/20

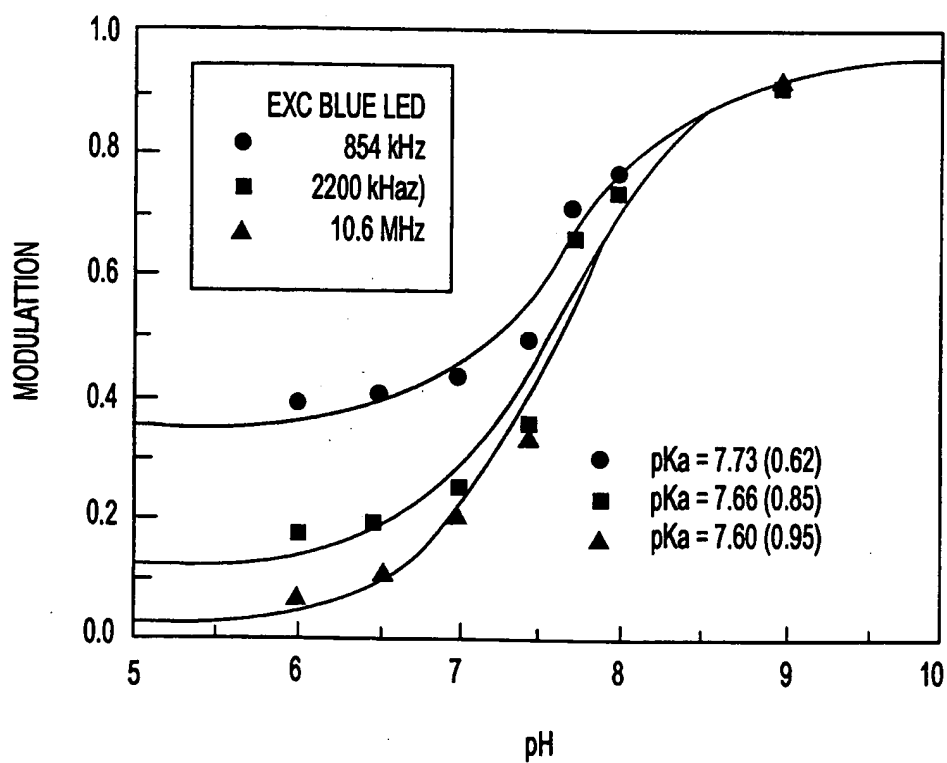


FIG. 15

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/11192

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 21/65

US CL : 436/ 172

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/ 172, 63, 74, 79, 80, 81, 82, 83, 84; 435/ 4, 7.21, 968; 250/ 200, 459.1; 524/ 408

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,866,430 A (GROW) 02 FEBRUARY 1999, see entire document.	1-2
Y	US 5,759,767 A (LAKOWICZ et al.) 11 October 1996, see entire document.	1-2
Y	US 5,696,193 A (DANIEL et al.) 09 December 1997, see entire document.	1-2
Y	US 5,648,270 A (KUHN et al.) 15 July 1997, see entire document.	1-2

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 JULY 1999

Date of mailing of the international search report

10 SEP 1999

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GAILENE R. LABEL

Telephone No. (703) 308-0196

**THIS PAGE BLANK (USPTO)**